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The design of a liver-selective form of interleukin-10

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Chapter - 2

The use of cytokines and modified cytokines as therapeutic agents: present state and future perspectives

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Abstract

Cytokine-based research is revolutionizing the treatment of several diseases including cancer, inflammation, infectious diseases, obesity, haematological disorders and other diseases affecting the immune system. Many of the original cytokine treatments have given way to more refined approaches as the knowledge and discovery process concerning complex cytokine networks becomes further clarified. However, the classical problem with the clinical use of cytokines is that the administration of these proteins must be by injection, either intravenously or subcutaneously. In addition, cytokines often have short plasma half-lives, due to rapid renal excretion and proteolytic degradation in plasma, whereas their activity on cells is usually most optimal after long exposure times. As a consequence, they have to be administered frequently, which makes them very expensive as a drug. Another problem is that the proteins have to be produced by recombinant techniques, and these recombinant proteins can be immunogenic when administered frequently. But most importantly, the clinical use of many cytokines is limited because of their pleiotropism. Because their receptors are expressed in a number of cells and tissues, systemic application of the cytokines can easily result in undesired effects. Despite several obstacles, numerous therapies with cytokines are continuously emerging. This demands to use better engineered cytokines in order to enhance their therapeutic effectiveness and decrease undesired effects. Chemical modifications with soluble polymers as well as carbohydrates have been used to improve the pharmacokinetics of various cytokines and to protect the cytokines from proteolytic degradation. A selective delivery of the cytokines to a specific organ or tissue is now also investigated. Chemical modifications of cytokines with specific carriers or receptor recognizing ligands could be a novel strategy to enhance the concentrations of the cytokines at the diseased target site, thus increase the potency of the cytokines, while reducing the concentration in the rest of the body thereby avoiding undesired effects. The current status of various cytokines and their modified forms as used nowadays in animal or patient studies to treat various diseases and the problems encountered with the therapeutic use of these proteins will be the subject of this review.

Cytokine and a concept of cytokine-based therapies

Cytokines are extracellular signaling-proteins necessary for cell-to-cell communication throughout the body. They are transiently produced by most cell type, and act through binding to specific plasma-membrane receptors, thereby inducing signal transduction pathways, which leads to activation of effector mechanisms within the responding cells. Cytokine-signaling plays an important role in both health and disease states. It is crucial during prenatal development and postnatal growth, remodeling and maintenance of every tissue and organ, and essential for inflammatory and immune responses as well as for wound healing and tissue repair.^[1-4]

Cellular communication by cytokines may occur at very short distance, for instance between two cells that are attached to each other, at moderate distance, within a small tissue area or even at long distance, acting systemically throughout the entire organism. The majority of immune responses are local.^[1,5]

Cytokines constitute a complex network and the specificity of this network is influenced by several factors: the expression of receptors, cell-cell interaction, short half-life of cytokines, and the existence of cytokine antagonists. Moreover, the synergistic and antagonistic effects that cytokines have on each other contribute significantly to the specificity of the response.^[5]

Cytokines are a large group of molecules (at least over 30 different) which are grouped together according to which class of molecules they belong and include the interferons (IFNs), interleukins (ILs), various colony-stimulating factors (CSFs), the tumor necrosis factors (TNFs) and transforming growth factors (TGFs). The ability of these molecules to regulate the functions of cells has stimulated many researchers to investigate their potential as therapeutic agents. Current status of cytokines approved by Food and Drug Administration (FDA) for clinical application is described in table I.

Table I. Cytokines licensed for clinical applications

Brand name (generic name)	Nature of agent	Approved application
Neupogen [®] (filgrastim)	G-CSF	Cancer patients receiving myelosuppressive chemotherapy; patients with acute myeloid leukemia receiving either induction or consolidation chemotherapy; cancer patients receiving bone marrow transplant; patients undergoing peripheral-blood-progenitor-cell collection and therapy; patients with severe chronic neutropenia
Leukine [®] (sargramostim)	GM-CSF	Following induction chemotherapy in older adult patients with acute myelogenous leukaemia; and following transplantation of autologous peripheral-blood-progenitor cells; to accelerate myeloid recovery after allogeneic bone-marrow transplantation
Roferon [®]	Interferon- α 2a	Hairy leukaemia, malignant melanoma, condylomata accuminata, AIDS-related Kaposi's sarcoma, chronic hepatitis B, chronic hepatitis C, follicular (non-Hodgkin's) lymphoma, chronic myelogenous leukemia
Intron A [®]	Interferon- α 2b	
Avonex [®] (interferon- β 1b)	Interferon- β	Relapsing forms of multiple sclerosis
Rebif [®] (interferon- β 1a)	Interferon- γ 1b	Chronic granulomatous disease, severe, malignant osteopetrosis
Actimmune [®]		
Betaseron [®]	Interferon- β 1b	Treatment of secondary progressive forms of multiple sclerosis
Proleukin [®]	IL-2	FDA approval for treatment in metastatic renal cell carcinoma (kidney cancer) in 1992, and for treatment in metastatic melanoma (a type of skin cancer) in 1998
Neumega [®]	Recombinant human IL-11	Prevention of severe thrombocytopenia and to reduce of the need for platelet transfusions following myelosuppressive chemotherapy in patients with nonmyeloid malignancies who are at high risk of severe thrombocytopenia
Tenovil TM	Recombinant human IL-10	Phase I clinical trials for rheumatoid arthritis, Crohn's disease, psoriasis treatments
Axokine [®]	Ciliary neurotrophic factor (CNTF)	Phase III clinical trial for obesity treatment

(original data from FDA, update: December 12, 2003)

Cytokine-based therapies

An increasing number of investigations and knowledge about the cellular mechanism of diseases have opened opportunities to search for more refined and rational approaches of therapy. Cytokines and growth factors are suggested to be involved in all important biological processes and thereby important in the regulation of many diseases. The pathogenesis of immune system-affected diseases generally involves a number of stages: initiation, perpetuation, tissue damage, repair, remodelling, and restoration of tissue damage, each of which involves different cell and molecular interactions.^[2-8]

The realization that cytokines have an enormous therapeutic potential in treating a variety of clinical conditions has prompted wide-spread cytokine research during the past decade. Consequently, a large number of new cytokines have been identified, cloned, and characterized and their physiological and pathophysiological roles became topics of intense investigation. The cloning and mass production of recombinant cytokine proteins opened a new world of therapeutic possibilities.^[9,10] Cytokines provide useful treatments of infections, and of cancer symptoms and diseases affecting the immune system.^[2,3,11] The use of cytokines so far in those diseases, their mechanism of action and current status in the therapy both in experimental animal models and in patients will be described in this review.

Cytokines used for cancer treatment

In principal, cytokines which are able to control the proliferation, differentiation, survival and effector functions of progenitor cells are potential anticancer agents.^[12] Many cytokines have demonstrated the ability to control these processes. These include IL-1, -2, -3, -4, -6, -7, -12, -18, tumor necrosis factor alpha (TNF- α), interferon (IFN) and granulocyte macrophage - colony stimulating factor (GM-CSF).^[12-25] Cytokines that have been approved by the FDA for the treatment of cancer are interleukin-2 (IL-2) and IFN- α 2b. Others, still under evaluation are GM-CSF, IL-12 and IL-18.^[25]

IL-2 is a powerful immunoregulatory lymphokine produced by T-cells in response to antigenic or mitogenic stimulation. IL-2 inhibits tumor growth indirectly by stimulating T-cells which recognize and kill the tumor. A therapy

involving IL-2 appears to be most suitable for metastatic melanoma, non-lymphoblastic leukemia and renal cell carcinoma.^[15,17,19,26,27] High dose (HD) bolus IL-2 received US FDA approval for metastatic renal cell carcinoma (1992) based on data that revealed durable responses in a small percentage of patients.^[27] However, this regimen is associated with significant toxicity and costs, which has limited its application to highly selected patients treated at specialised centers. Several investigators have evaluated regimens with lower doses of IL-2 in an attempt to decrease toxicity. Attempts were also made to improve treatment efficacy by adding interferon alpha followed by 5-fluorouracil to low-dose IL-2 regimens. These regimens were reported to produce response rates and survival comparable to HD IL-2 with much less toxicity, but possibly fewer durable responses.^[26-29] Based on positive preclinical data, other cytokines (e.g., IFN and IL-12) have also been given to patients with metastatic renal cell carcinoma but with limited success.^[29,31] The efficacy and toxicity studies of IL-2 and other cytokines in patients with renal cancer are, at present, ongoing in phase III trials which can help to define the proper use of these agents in this disease.

The IFNs have been found to produce a large array of molecular and cellular actions of potential relevance to their use in tumor.^[32] The antitumor effects of the IFNs result from a combination of direct cytotoxicity to tumor cells and from stimulation of natural killer cells and macrophages. IFNs have demonstrated activity in a broad range of malignant disorders.^[32-35] In recent years, their use in cancer chemotherapy has grown as FDA has approved the use of IFN- α 2a and IFN- α 2b for the treatment of AIDS-related Kaposi's sarcoma, hairy cell leukemia and chronic myelogenous leukemia. In the treatment of other neoplasms it is still in the stage of clinical development.

GM-CSF is a hematopoietic factor that stimulates the development of neutrophils and macrophages and promotes the proliferation and development of early erythroid megacaryotic and eosinophilic progenitor cells. GM-CSF has been approved for use in stem cell and bone marrow transplants, and has been suggested to be of use against melanoma through the stimulation of antigen-presenting cells.^[36-39]

Recombinant human interleukin-18 (rHuIL-18), a newly identified cytokine (in 1995), is currently in clinical trials for treatment of cancer. Herzyk DJ *et al*^[24,25] reported that results of preclinical toxicity and safety studies with rHuIL-

18 in cynomolgus monkeys and recombinant murine IL-18 (rMuIL-18) in mice showed that rIL-18 was well tolerated at pharmacologically active doses in both monkeys and mice.

Cytokines used for the treatment of autoimmune diseases

Autoimmune diseases are receiving increasing attention in the pharmaceutical industry as progress is made in the understanding of underlying immune and inflammatory processes. The pathogenesis is generally initiated by the activation of peripheral T-cells. The activation of peripheral T-cells by foreign and self-antigens is under stringent control by different mechanisms, both thymic and peripheral, and a failure in their control mechanisms may lead to autoimmune diseases.^[40,41] To interfere selectively with the activation of pathogenic T-cells, immunosuppressive therapy can be directed to three cellular targets: antigen-presenting cells, autoreactive T-cells and suppressor/regulatory T-cells, with the common goal to selectively inhibit the activation of pathogenic class II-restricted CD₄⁺ T-cells.^[40] Cytokine-based manipulation offers unique possibilities to interfere with autoimmune diseases and anti-inflammatory cytokines like IFN- β , TGF- β , IL-4 and IL-10 are being studied now.^[40,41] The most common examples of autoimmune diseases such as multiple sclerosis, psoriasis, rheumatoid arthritis, inflammatory bowel disease, and insulene-dependent/juvenile type I diabetes mellitus will be discussed briefly in this section.

Cytokines used for multiple sclerosis treatment

Multiple sclerosis (MS) is a chronic autoimmune disease of the central nervous system in which an abnormal immune response damages the myelin-covered sheaths that surround and insulate nerve fibers in the brain and spinal cord. Immune responses are thought to contribute to the damaged myelin in people with MS.^[42] The effects of MS can be debilitating and may severely affect a patient's quality of life. In severe MS, patients have partial or complete paralysis on a permanent basis. Three interferon beta medications have been approved to date by the FDA for treating relapsing MS: IFN- β 1a (Avonex[®] and Rebif[®]) and IFN- β 1b (Betaseron[®]). IFN- β has multiple effects on the immune system, including increasing the activity of suppressor lymphocytes and inhibiting stimulation of

other immune cells.^[40,43,44] The net effect of all these actions is an attenuation of the immune response thereby slowing the progression of this disease.

Cytokines used for psoriasis treatment

Psoriasis has recently been established as an autoimmune disease, which means that T-cells of the immune system recognize an antigen in the skin and attack the areas where that antigen is found.^[45] Infiltration of T-cells seems to be the primary event that precedes the keratinocyte hyperproliferation. It is suggested that systemic lymphocyte activation is followed by the local accumulation of specific CD₄⁺ T-cells and subsequently by the activation of intradermal CD₈⁺ T-cells. So far, it seems that CD₄⁺ T-cells create an appropriate type-1 cytokine environment for CD₈⁺ T-cells activation that eventually trigger the psoriatic cascade. Thus, T-cells are responsible for initiation and maintenance of psoriasis.^[46-48] It is not fully understood what causes this immune response, but it could be triggered by a number of factors such as systemic infections, stress or hormonal changes, injury to the skin (sunburn or surgery incision), alcohol, smoking, obesity, poor diet, and certain medications.^[49] As psoriasis is a chronic relapsing disease, intermittent treatment may span a lifetime.

In the first clinical trials in patients with established psoriasis, IL-10 showed moderate antipsoriatic effects and was well tolerated.^[50] IL-10 can influence T1/T2 differentiation, antigen-presenting cell functioning, antigen-presenting cell-mediated T-cell activation, and T-cell, B-cell, and mast cell growth and differentiation that is aberrant in various disease processes. A long-term application in psoriatic patients remission showed that IL-10 therapy decreases the incidence of relapse and prolongs the disease-free interval. The immunological effects observed during these clinical studies together with *in vitro* observations suggests that IL-10 exerts its antipsoriatic activity by effects on different cell populations including antigen presenting cells and T-cells (lasting type 1/type 2 cytokine balance shift), but not through direct effects on keratinocytes.^[51-54] Although IL-10 seems to have a major role in psoriasis, further investigations are still required to fully determine whether IL-10 application will become a successful anti-psoriatic therapy.

Cytokines used for rheumatoid arthritis treatment

Rheumatoid arthritis (RA) is a chronic progressive inflammatory disease of multifactorial aetiology. This disease is one of the more common autoimmune diseases in the western society and difficult to treat.^[55,56] This elicits a great deal of interest in the search for novel drugs to treat this condition. Because of the emerging acceptance of RA as an autoimmune disease, much of the current therapeutic research has focused on immune mediators associated with the development of persistence of this disease. One such mediator is TNF which is known to be one of the pivotal factors in initiating and maintaining the inflammatory cascade.^[55-58]

As potent mediators of immune response, the interleukins are also being considered as potential targets in RA treatment. IL-6, a cytokine with both pro- and anti-inflammatory actions, is involved in T-cell and B-cell growth. Recent research has shown that transcription of IL-6 genes may be regulated by IL-1 suggesting a possibility to regulate IL-6 production in RA patients. Obviously, this production has to be controlled locally.^[56]

IL-4 and IL-10 are anti-inflammatory cytokines that suppress the release and block actions of TNF- α , IL-1 and IL-6.^[58-60] Recombinant IL-4 and IL-10 are currently under investigation in clinical trials in RA treatment. IL-11 is also gaining some attention for the treatment of this disease. IL-11 has been shown to indirectly decrease production of TNF- α and enhance the production of IL-10 thus attenuating the inflammatory process.^[61-63] Moreover, IL-11 has shown to reduce the activity of MMP-1 and MMP-3 which are collagenases responsible for the breakdown of joint tissues.

Cytokines used for inflammatory bowel disease treatment

Inflammatory bowel diseases (IBD), such as ulcerative colitis and Crohn's disease, result from an interaction between susceptibility genes, the host's bacterial environment, gut barrier defects, and immunological factors.^[64] Recent evidence suggests that a pathologic activation of the mucosal immune system in response to antigens is a key factor in the pathogenesis of IBD. The pathogenesis of IBD is characterized by an imbalanced activation of Th1- and Th2-lymphocytes.^[64-66] Several new therapeutic strategies are currently being tested in clinical practice, including recombinant anti-inflammatory cytokines (IL-4, IL-10,

IL-11 and IFN- α).^[66-79] It has recently become apparent that these cytokines have better short-term effects than conventional drugs, and they could change the treatment strategy of IBD in the near future.

IL-10 has a major role in the regulatory network of cytokines controlling mucosal tolerance, and it is, therefore, not surprising that this cytokine is proposed as a potent anti-inflammatory biological therapy in chronic IBD. The effectivity of IL-10 in animal models of colitis is quite promising.^[67-72] In the *in vitro* system, IL-10 downregulates the enhanced proinflammatory cytokine release from lamina propria mononuclear cells isolated from patients with Crohn's disease. Furthermore, clinical trials using recombinant human IL-10 (rhIL-10) for the treatment of patients with Crohn's disease have been published. A double blind controlled trial to evaluate the safety and tolerance of rhIL-10, Tenovil™, for 12 consecutive weeks indicated that this drug was safe and well tolerated.^[68] The therapeutic effect of systemic administration of IL-10 in patients with IBD, however, has not been satisfactory. Despite the disappointing results of IL-10 therapies so far, there is still enough rationale for the use of IL-10 as an anti-inflammatory biological drug in chronic IBD.^[70-72] Therefore, some novel alternative approaches including improvement of the formulation technology and delivery systems of this cytokine are investigated in mouse models.^[72] Other approaches including IL-10 gene therapy and the use of genetically modified bacteria are also under investigation. Both latter novel therapies have been shown to be successful in animal models of disease, and clinical testing is currently underway.

A tissue-protective effect of IL-11 in the intestinal mucosa can be deduced from animal models for IBD as well. The clinical usefulness of this anti-inflammatory cytokine is therefore currently investigated in patients with IBD.^[75-76]

Cytokines used for immune-mediated or type I diabetes mellitus treatment

Type 1 diabetes mellitus results from insulin deficiency caused by autoimmune destruction of the insulin-producing beta-cells in the pancreatic islets of Langerhans.^[80] The autoimmune response against islet beta-cells is believed to result from a disorder in the immunoregulation. Th1 cell activation and cytokine production shift the balance between Th1 and Th2, favoring the up-

regulation of proinflammatory activity that leads to destruction of insulin-producing pancreatic beta cells in type 1 diabetes.^[80-82]

Administration of IL-10 prevents autoimmune diabetes in non-obese diabetic (NOD) mice.^[83-84] A single systemic administration of adeno-associated viral (AAV) vIL-10 significantly reduced insulinitis and prevented diabetes development in NOD mice. This protective effect correlated with sustained transgene expression and protein production. Moreover, splenocytes from the treated mice blocked diabetes transfer to NOD recipients, suggesting that vIL-10 induces an active suppression of autoimmunity. In addition to the use of IL-10 for this disease, a therapy with IFN- α is also currently in clinical trial phase II.^[85-86] IFN- α is able to enhance B cell survival and prolong the period in which the insulin need becomes minimal and glycemic control improves.

Antiviral cytokines

A family of cytokines which has an antiviral activity is the interferon family. The target of interferon is the cell rather than the virus itself. Through binding to specific receptors on the cell surface and subsequent activation of specific genes, interferons induce an antiviral state which makes cells less permissive for virus replication. The antiviral state is composed of various antiviral mechanisms which seem to act independently and show some specificity for different viruses. Translational control affecting viral protein synthesis may be the most common mechanism through which virus replication is inhibited. There is evidence that antiviral and antigrowth activities of interferons share similar mechanisms.^[33,87-88] The use of cytokines as an antiviral therapy is described in this section.

Antiviral cytokines used for Human Immunodeficient Virus treatment

The current understanding of the immunopathogenesis of human immunodeficient virus (HIV) disease and the deficiencies of highly active antiretroviral therapy (HAART)-induced immune restoration have formed a basis for the use of cytokines for HIV therapy.^[89] This is due to the function of the cytokines, that is, enhance and preserve immunity, control mobilization of latent reservoirs as part of a viral-eradication strategy, potentiate vaccine-induced responses, and inhibit directly the HIV replication.

Antiviral cytokines used for hepatitis C virus infection treatment

In the majority of cases hepatitis C virus (HCV) infection gives rise to an acute illness; 80% of such cases develop into chronic hepatitis. Almost all patients develop a vigorous antibody and cell-mediated immune response which fails to clear the infection but may lead to liver damage. Most flavivirus infections are cytopathic, but this has not been directly tested in the case of HCV since the virus cannot be cultured. Spontaneous resolution of chronic liver disease is very rare (<2%) and patients with chronic disease are at risk of developing liver cirrhosis and eventually hepatocellular carcinoma (HCC). The duration from the onset of acute hepatitis until the time of diagnosis of cirrhosis or HCC is about 20 to 30 years. The acute phase lasts from the onset of disease until 2-3 years thereafter, and the silent phase which follows lasts for 10-15 years. Since so little is known about the biology of HCV, it is presently unclear how this RNA virus establishes a persistent infection.^[102-103]

At the present time, the only cytokine therapy with any demonstrated efficacy against HCV-induced liver disease involves the use of IFN- α , but this approach is not entirely successful. The current standard combination of interferon-based therapies and ribavirin is effective in only 50% of patients. In addition, this combination is expensive, requires lengthy periods of administration, and is associated with significant side effects.^[104] Nevertheless, careful and timely management of side effects, which are experienced by all patients, may improve adherence to antiviral therapy and further improve response rates.

Anti-inflammatory cytokines

The anti-inflammatory cytokines are immunoregulatory molecules that control the proinflammatory cytokine response. Their physiologic and pathologic role in inflammation is increasingly recognized. Major anti-inflammatory cytokines include IL-1 receptor antagonist, IL-4, IL-6, IL-10, IL-11 and IL-13.
^[2,3,9,11,40,58,59,61,62,105,106]

Table II. Cytokines in HIV-infection: actions and stage of therapeutic development in HIV infection.

Cytokine	Potential therapeutic role in HIV	Preclinical and phase I – III trial data	Ref.
IL-1	Enhances responses to IL-2 and IL-15, reduction in HIV replication, reduced activation-driven T-cell apoptosis	Preclinical	90,9
IL-2	<ul style="list-style-type: none"> - Preservation of the CD₄⁺ T-lymphocytes - Combined with protease inhibitor to treat an early HIV infection, combined with anti- HIV agent treat HIV positive patients - Combined with anti-HIV agent to treat HIV infection in children with weakened immune system 	<ul style="list-style-type: none"> - Phase II data - Phase III - Phase I/II trials as a vaccine adjuvant ongoing Phase I: vaccine adjuvant 	*
IL-7	Reverses CD ₄ ⁺ T-cell lymphopenia, enhances T-cell-based vaccine responses	Primate studies using IL-7 as an immunotherapeutic and as an HIV T-cell vaccine adjuvant are ongoing	92
IL-12	- Combined with liposomal doxorubicin for treating Kaposi's sarcoma in people infected with HIV	- Phase II	*
IL-15	Enhances CD ₈ ⁺ T-cell function and HIV-specific immunity	Preclinical, proposed phase I as a vaccine adjuvant	93,94
IFN- α and PEG IFN- α	Enhances CTL responses; direct antiviral effect	<ul style="list-style-type: none"> - Phase II pre HAART - [PEG]IFN-α2β, phase I/II as an adjuvant to ART - Pilot study as monotherapy in HAART-naïve HIV-infected patients 	*, 95 96
IFN- γ	Enhances CTL responses Improves phagocytic response to intracellular pathogens	Phase II/III study performed pre HAART	97
GM-CSF	Enhances macrophage function	Phase II/III	98,99
G-CSF	Increases myeloid precursors, Enhances <i>in vitro</i> IL-2 production Increases CD ₄ ⁺ T-cell level	Phase II/III	100,101

* Data were adopted from clinical trials inspired by National Institutes of Health, USA; website: www.clinicaltrials.gov

Anti-inflammatory cytokines used for asthma treatment

Asthma is a complex inheritable inflammatory disorder of the airway in which the development of clinical disease depends on the environmental exposure. The recognition that several cytokines have inhibitory effects on allergic inflammation and that some of them might be deficient in patients with asthma suggest that they have the potential to provide new and more specific approaches for therapy.^[107-110]

It has been well established that T helper type 2 (Th2) lymphocytes and their cytokines have an important role in allergic asthma. IL-4 has the potential to play a key role in asthma through its ability to induce differentiation of naive Th0 cells to Th2 lymphocytes, which express several cytokines including IL-5 (eosinophil growth factor), IL-9 (induces mucus expression), and IL-13 (induces airway hyperreactivity).^[111-114] Thus, inhibiting IL-4 would also inhibit additional cytokines expressed by Th2 cells.

Preclinical studies in animal models demonstrate that IL-12 inhibits allergen sensitization, as well as allergen-induced inflammation. Studies in humans with mild asthma have demonstrated that intravenous infusion of IL-12 was effective in inhibiting eosinophilic inflammation but not airway hyperreactivity.^[115-117] A major action of IL-12 is to induce the development of Th1 cells, while suppressing Th2 cells. It is likely that IL-12 plays a critical role in determining the balance between Th1 and Th2 cells, thereby inhibiting IgE synthesis and allergic inflammation. Other inhibitory cytokines such as IL-10 and IFNs also have been used in clinical trials for the treatment of chronic airway diseases like asthma. IL-10 and IFN- γ are considered as potential therapeutic agents for bronchial asthma in which eosinophil accumulation plays a major pathogenic role.^[118-123]

Anti-inflammatory cytokines used for glomerulonephritis treatment

Glomerulonephritis (GN) is a type of kidney disease caused by inflammation of the internal kidney structures (glomeruli). This disease may be a temporary and reversible condition, or it may be progressive. Progressive glomerulonephritis may result in destruction of the renal glomeruli and chronic renal failure and end stage renal disease. The disease may be caused by specific problems with the body's immune system, but the precise cause of most cases is

unknown. Current therapeutic approaches with conventional drugs are not satisfactory since long-term therapy induces side effects to non-target organs. Interest has recently focused on anti-inflammatory cytokines such as IL-4, IL-10 IL-13 and low dose of TGF- β .^[124-125] In experimental models of auto-immune anti-GBM nephritis, early administration of IL-4 or IL-10 reduces proteinuria, macrophage infiltration and crescent formations. In acute mesangial proliferative GN induced by antithymocyte antibody, transfer of a mutated gene that encodes active TGF- β suppresses mesangial cell proliferation. The potent effects of these cytokines in the deactivation of Th1 cells, macrophages and mesangial cells limit the progression of this disease.^[124,126-128]

Antifibrotic cytokines

Fibrosis is a pathologic process, which includes scar formation and over production of many extracellular matrix components, as a response to chronic tissue damage.^[129] The fibrogenesis includes interaction between many cell types and cytokines, and when the balance becomes profibrotic, scar tissue formation is promoted.^[129-134] Major profibrotic agents are type 2 CD₄⁺ lymphocytes, CD₄₀ receptor and ligand interaction, and the following cytokines: IL-4, TGF- β and platelet derived growth factor (PDGF). Fibrosis is a reversible process, and is usually treated with anti-inflammatory and immunosuppressive agents. This kind of therapy is not proven successful in reversing the fibrotic process, but mostly focuses on the treatment of symptoms and complications and sometimes it harms more than it cures. Many patients suffer from fibrotic diseases and therefore, the development of antifibrotic agents which are targeted to the pathologic molecular processes are really required. The major antifibrotic cytokine that is being used in clinical trials is IFN- γ since it inhibits collagen synthesis in fibroblasts.^[135] The effect of IFN- γ on collagen synthesis by arecoline-stimulated OSF (oral submucous fibrosis) fibroblasts *in vitro* and the effect of intra-lesional IFN- γ on the fibrosis of OSF patients has been investigated.^[135,136] The results showed that IFN- γ exerts a positive effect in both conditions. The antifibrotic potential of IFNs on non-viral diseases has been tested in different experimental models of hepatic fibrosis.^[137,138] These studies showed significant effects on the fibrotic process.

Moreover, the use of IL-10 in patients with advanced fibrosis (HCV-related liver fibrosis) who had failed antiviral therapy with IFN is currently under

investigation.^[139,140] The preliminary data revealed, that 50% of IL-10-treated patients showed a decrease on the inflammation and fibrosis score, which indicates that this cytokine is a promising candidate as a therapeutic agent in this disease. However, since IL-10 also has an immunosuppressive action, the toxicity study of the long-term native IL-10 therapy for the treatment of HCV-associated liver fibrosis is still required to determine whether IL-10 is applicable in this chronic disease.

Problem of the use of cytokines as therapeutic agents

The clinical use of cytokines is hampered partly because of common drawbacks of the use of proteins as therapeutic compounds. This creates problems with respect to the production, the stability, the purity and the route of administration. On top of that, the poor stability *in vivo* and short half-life of cytokines create problems because their activity on cells is usually most optimal after long exposure times. These proteins are however rapidly cleared from the blood by the liver, kidney and other organs. Clearance mechanisms include glomerular filtration, bile excretion, receptor binding and/or enzymatic degradation. Plasma proteases cause degradation and rapid loss of biological activity. Therefore, achieving a clinical effect is still a problem. In order to improve the efficacy of cytokines, they require repeated administration. Initially, continuous infusion (CI) was used to address this pharmacological deficiency. CI has the advantage of administering drugs in a controlled manner and this is particularly appropriate when it is important to maintain constant plasma concentrations. However, the requirement for continuous venous access and the use of ambulatory pumps obviously limits their use. In addition, wider use of cytokines in the clinic has been hampered by the pleiotropic nature of such factors leading to marked toxicities following systemic administration. On the molecular level, this pleiotropism can be explained through an understanding of the receptor system specific for each cytokine and the cells on which they are expressed.^[5,141,142] The receptors for most cytokines are distributed throughout the body, and found on many different cell types accounting for many non-related actions. Low targeting efficiencies to the site of action may thus be the most important clinical problem after systemic administration of exogenous cytokines.

In some cases, activities responsible for side effects have been attributed to discrete areas of the proteins and “structure driven design” can be used to generate novel cytokines with a better clinical profile. In other cases, structural alterations can enhance activity by improving the pharmacokinetic profile, proteolytic stability, and biodistribution.

Mostly, cytokines used for therapeutic purposes are produced through recombinant technology which allows mass production. Therapeutic recombinant human cytokines currently in clinical use as listed in table I include interferons (IFN- α , IFN- β and IFN- γ), interleukins (IL-2, IL-10 and IL-12) and hematopoietic factors such as granulocyte colony-stimulating factor, granulocyte-macrophage colony-stimulating factor, erythropoietin and thrombopoietin. Their use as therapeutic agents has been challenging since the safety and efficacy of these products are complicated by immunogenicity issues as well.^[143-145]

Modification of cytokines

Considering the many problems encountered in the use of cytokines for therapeutic purposes outlined in the previous section, several approaches that can enhance the therapeutic effectiveness of these compounds are being developed. Some of these strategies have been devised to retard the clearance of these cytokines by means of polyethylene glycol (PEG) coupling. To that end, these cytokines have been chemically modified by covalent attachment of PEG. Table III shows several cytokines with clearance-retarding modifications currently in clinical use. Other clearance-retarding modifications currently applied are coupling to biodegradable polymers^[146,147], sugar chains^[148] or protein-protein cross-linking.^[149] Genetic modification has also been employed to fuse cytokines of interest with long-lived plasma these proteins.^[150] The examples of cytokines modified with these techniques are described in more detail in the next sections.

While all modifications may reduce the biological activity of the cytokines of interest or elicit antibody formation in recipient animals or patients, there are now sufficient experiences in this area to create an optimal clearance-extending strategy. With the explosive growth of genomic and proteomic information, an exponentially increasing number of engineered cytokines are likely to be developed. In addition to the modifications that change the pharmacokinetics and/or physicochemical properties of the molecule, modifications with selective

carriers or receptor recognizing ligands to deliver potential cytokines to the target site of disease have been developed as well.

Modification to improve the physicochemical properties and the pharmacokinetic profile of cytokines

Pegylation

Pegylation, the technology of polyethylene glycol (PEG) conjugation to proteins, holds significant promise in maintaining effective plasma concentrations of systemically administered cytokines that might otherwise be hampered *in vivo* by rapid elimination by the kidneys.^[146,147]

Table III. Pegylated-cytokines used in clinical application and their stage of development

PEG-cytokine	Indication for	Status
PEGASYS® (PEG-interferon- α 2b)	Treatment of adults with chronic hepatitis C who have compensated liver disease and who have not been previously treated with IFN- α	FDA approved in 2002 (on the market)
	Patients with stage IV melanoma	Phase II
	Combination therapy with Ribavirin, USP (Copegus), for the treatment of chronic HCV infection	FDA approved in 2002 (phase IV)
PEG interferon alpha-2b*	Metastatic kidney cancer	Phase II
PEG-Intron® (PEG-interferon- α 2b)	Treatment of chronic hepatitis C in patients not previously treated with IFN- α who have compensated liver disease and are at least 18 years of age	FDA approved in 2001 (on the market)
PEG-IL-2*	HIV positive patients	Phase I
PEG-IL-6	Enhancement thrombopoietic activity	Animal trial
PEG-TNF- α	Anti-tumor activity	Animal trial
PEG-GM-CSF	Mobilization hematopoietic stem cells	Animal trial

* National Institutes of Health - USA

Attaching PEG molecules without obstructing the active sites that are essential for the cytokine effects is a major challenge in pegylation.

Current pegylation technology uses linkerless conjugation methods to allow coupling without added toxicity or immunogenicity, and to keep the innate surface charge of the pegylated molecule intact.^[146,151] In addition to controlling the size and complexity of PEG molecules, the attachment site can be manipulated to avoid steric hindrance of the cytokine's active receptor-recognition or substrate-interaction site. A few pegylated cytokines have been engineered to have an improved pharmacokinetic profile with preserved bioactivity (table III). They often have prolonged steady-state plasma concentrations *in vivo*, thereby making a reduced number of doses possible. Maintaining drug concentrations at or near target cells for an extended period of time is often clinically advantageous, and is particularly useful in antiviral therapy (e.g. PEG-interferons), since constant antiviral pressure should prevent replication. This is particularly important to suppress the emergence of resistant variants. Additionally, PEG modification may decrease adverse effects caused by the large variations of plasma drug concentrations associated with frequent administration and by the immunogenicity of unmodified proteins.

Modification of cytokines with another biodegradable polymer

Development of polymeric molecules that can be useful as a drug delivery system, by regulating the characteristics of drugs *in vivo* is now also intensely investigated. Since the molecular structure of PEG does not readily allow the addition of new functions, other polymeric modifiers to which new functional groups can be attached are required. Polyvinyl pyrrolidone (PVP) is a novel polymeric modifier for polymer-conjugated cytokines, and its efficiency and applicability as a drug delivery system (DDS) was evaluated.^[152,153] PVP has been used for covalent conjugation of IL-6 as well as TNF- α .^[154,155] PVP-conjugated IL-6 showed more than 50-fold greater thrombopoietic potency *in vivo* than native IL-6. No side effects, such as body weight loss, were observed in the M-PVP-IL-6 treated mice.^[155] Likewise, conjugation of PVP-TNF- α was also able to increase the TNF- α half-life and to selectively increase antitumor potency of this cytokine in a mice model.^[154]

Carbohydrate modified-cytokines

To improve the pharmacokinetic properties of recombinant IL-2, modification of this cytokine with various carbohydrate moieties (glycosylation) is currently also studied. The rIL-2 is glycosylated at least one of the lysine residues in rIL-2. The carbohydrate to be linked to rIL-2 is conveniently selected from the group consisting of monosaccharides and oligosaccharides. Various mono- and oligosaccharide-tether-conjugates have been attached chemically via an amide bond to the amino group of one or more of the 11 lysines in rIL-2. Glycosylated rIL-2 is chemically more stable relative to unglycosylated rIL-2. In addition, the recovered glycosylated rIL-2 was also significantly more soluble than native rIL-2 while it retained its biological activity. Surprisingly, several glycosylated rIL-2 preparations which were prepared by this method lost most of their T-lymphocyte activating ability, while retaining most of their other biological activities, i.e., the ability to enhance NK (natural killer) cell and LAK (lymphokine activated killer) cell (cells that kill tumor cells but not normal cells) activities. Carbohydrate-modified rIL-2 as an antitumor agent is now being tested in phase I clinical trials.^[Dupont Technology Bank, <http://dupont.t2h.yet2.com/t2h/page/homepage>]

Modification of cytokines for targeting purposes*Antibody-cytokine fusion protein*

Antibody-cytokine fusion proteins, which in common terms are called immunocytokines, are fusion proteins consisting of an antibody attached to a cytokine.^[156] These molecules combine the specificity of an antibody with the powerful immune-stimulating features of cytokines. The use of antibody-cytokine fusion proteins represents a method for delivering therapeutic cytokines to specific sites. Immunocytokine constructs were firstly designed for tumor targeting. Immunotherapeutic approaches using monoclonal antibodies are based on the concept of targeting tumor-associated antigens that are expressed to a greater extent on the surface of tumor cells than on normal cells or tissues. This approach overcomes the problems that are associated with the individual uses of cytokines. First, the conjugation of the cytokine to the mAb enables localization to the tumor, thus avoiding high-dose systemic administration. Second, the large size and inherent stability of the mAb increases the half-life of the cytokine,

enabling it to exert its actions without rapid clearance.^[157,158] Finally, the antitumor immune response elicited by the antibody itself is enhanced as a result of the enhanced local concentration of the cytokine.

Immunocytokines do not cause the side effects of conventional chemotherapy. Most cancer chemotherapy agents kill dividing cells, both normal and cancerous, so that the immune system, the intestine and stomach, skin, and hair are damaged. One successful application of this construct in animal models is IgG anti-idiotypic(Id)-IL-2 fusion protein. This chimeric fusion protein was more effective for the treatment of B-cell lymphoma in mice than a combination of anti-Id Ab and IL-2. Additionally, co-administration of IL-2 with tumor-associated antigen (TAA)-specific mAbs has been demonstrated to enhance the anti-tumor response compared to either agent alone.^[159,160] This has led to the development of antibody-cytokine fusion proteins as novel anti-tumor therapeutics, with the antibodies in these immunocytokines targeted against TAAs.^[161] During the past decade, several groups have developed immunocytokines, including mAbs fused to TNF- α , IL-2, granulocyte macrophage colony stimulating factor (GM-CSF) and IL-12.^[162-165]

Another immunocytokine is Ab-IL-12 fusion protein which may be an effective alternative to systemic administration of IL-12 for the treatment of metastatic breast cancer. Using the tumor-targeting ability of the Ab, it should be possible to achieve effective local IL-12 concentration at the sites of tumors and metastases with lower doses of IL-12, thus decreasing the risk of toxicity associated with IL-12 treatment. An anti-Her2/*neu* mAb has also had success in clinical trials for the treatment of Her2/*neu*-expressing metastatic breast cancer.^[165] Fusion of a cytokine-like IL-12 that has anti-tumor and anti-metastatic properties to a Her2/*neu*-specific Ab may enhance its efficacy, particularly if it elicits a tumor-specific immune response.

Cytokine fusion proteins

Fusion proteins are emerging as a promising approach for targeting cytokines to the target site of a disease in order to generate an effective response as well as to increase the half-life of these proteins.^[166] The fusion cytokine approach has been developed for many cytokines: IL-10, IL-3, IL-2 and others.^[167]

Interleukin-10 (IL-10) is a cytokine with immunoregulatory properties that could potentially be harnessed for the treatment of inflammatory disorders like septic shock, or as adjunct therapy to prevent graft rejection. An IL-10/Fc fusion protein has been investigated in murine models of these two clinical situations because of the rapid clearance and short duration of effect of unmodified IL-10.^[168,169] The Fc portion of the chimeric protein was mutated in order to eliminate its cytolytic properties by altering four specific residues in the complement binding and Fc receptor-binding pockets (designated Fc--). *In vivo*, the IL-10/Fc-- protein was as effective as unfused IL-10 in increasing the survival rate of mice when administered thirty minutes prior to endotoxin injection, and much more effective than unfused IL-10 when administered the day before endotoxin. However, the fusion protein appeared to accelerate allograft rejection in a murine transplant model. Thus, inactivating the Fc and reducing its role into a simple means of preventing glomerular loss of the cytokine was warranted in one situation, but not the other. For comparative purposes, further investigations of an IL-10/Fc fusion protein with full Fc effector functions are still required.^[168]

The novel fusion protein DT(388)IL3, composed of the catalytic and translocation domains of diphtheria toxin (DT(388)) fused with a Met-His linker to human interleukin-3 (IL-3), was tested for antileukemia efficacy in an *in vivo* model of differentiated human acute myeloid leukemia (AML).^[170] DT(388)IL3 fusion protein demonstrates *in vivo* antileukemia efficacy and warrants further preclinical development for treatment of chemo-resistant, IL-3 receptor positive AML patients.

Another alternative strategy to improve the pharmacokinetic profile of cytokines is using human serum albumin (HSA) as a genetic fusion partner.^[149,171,172] This approach recently has been studied to create a new drug with combined biological properties of IL-2 and HSA (albuleukin). Albuleukin, has a significantly extended half-life, has proved to be an effective agent for suppressing tumor growth in mice, and is currently being evaluated in a phase I study in patients with solid tumors.^[173] The study performed recently supports the hypothesis that Albuleukin targets tissue where lymphocytes reside to much greater extent than does IL-2, and suggests that Albuleukin may exhibit improved efficacy and reduce toxicity in the treatment of solid tumors.

Carrier/ligand-modified cytokines for selective delivery of cytokines

Tissue selectivity of exogenous cytokines could be one of the stumbling blocks that hinder their clinical application. The specific delivery of these compounds to the target site, therefore, would be necessary to overcome such problems. The components which have been recognized to be tools for targeting purposes include receptors and ligands, where the receptors act as molecular targets or portals, and ligands, with receptor specificity and selectivity, are trafficked and routed to the target site. The use of carriers or bioligands to target therapeutic cytokines offers enormous options and opportunities through carrier construct engineering and could become a future reality in clinical practice.

Recently, a novel hepatic targeting system by using artificial ligand [(Gal)₃] for the asialoglycoprotein (ASGP) receptor has been developed for IL-2 to treat hepatic tumors in an animal model. This (Gal)₃-IL-2 shows a great potential with improved targeting efficiency to the liver, while the IL-2 activities are preserved, and the endocytosis via the ASGP receptor is avoided. The (Gal)₃ ligand increases the antitumor activity of IL-2 by enhancing its exposure to the surface of tumor cells in the liver.^[174]

Specific ligands-associated cytokines are also developed for TNF- α . An aminopeptidase N (CD13) or CNGRC peptide, a marker of angiogenic vessels, has been used for targeted delivery of TNF- α . This targeted system of TNF- α conjugated to CNGRC peptide improved the therapeutic index of this cytokine in tumor-bearing mice.^[175,176] In addition, a specific vascular delivery of TNF- α by conjugation to the ACDCRGDCFCG peptide, a ligand of α_v integrins, another marker of endothelial cells, is now also investigated. This ACGDRGDCFCG-mouse TNF conjugate showed an improvement of anti-neoplastic activities in tumor-bearing mice as compared to native TNF- α .^[176] Ligand-targeted therapeutics are successful means of improving the selective toxicity of anticancer therapeutics. For radioimmunotherapy, an immunotoxin and an immunoconjugate have received clinical approval and over 100 ligand-targeted therapeutics are currently in clinical trials as recently reviewed by Allen *et al.*^[177]

Currently, in our laboratory we are investigating a liver specific form of IL-10 (M6PIL-10). We aim to target this conjugate to the liver for the treatment of liver fibrosis. This construct is targeted to the insuline-like growth factor II/mannose 6-phosphate (IGF-II/M6P) receptor which is highly expressed on

activated hepatic stellate cells. These myofibroblast-like cells are crucial cells in the pathogenesis of liver fibrosis and IL-10 appears to be able to attenuate the fibrogenesis activities of this cell type. However, systemic administration of IL-10 caused unwanted effects. Our preliminary results show that the modified IL-10 retains the pharmacological activities of IL-10 in *in vitro* systems. In addition, M6PIL-10 did not accumulate in non-target organs, in particular in the kidney, while the concentration on the target site within the liver was enhanced. These results give a hopeful perspective to prevent the drawbacks of the use of unmodified IL-10 for the treatment of HCV-associated liver fibrosis that previously have been reported.^[139]

Conclusion and future perspectives

The use of recombinant gene technology to produce commercially available amounts of cytokines heralded an era of clinical uses of these agents. Although the response rates to cytokine therapies are modest and sometimes occur at the expense of great costs and toxicity, they are proof-of-principal that some serious diseases described in previous sections can be overcome by purely immune modulation. The interleukins and the interferons have been studied widely in various phases of clinical trials. Some trials are indeed still disappointing, because systemic administration of these cytokines causes the major problems induced by their short half-life and adverse effects.

Structural modifications as well as novel delivery systems of these proteins are currently under development to combat the obstacles as encountered by the use of native cytokines or conventional delivery of these agents. Some modifications of these cytokines are already approved by the FDA and are available on the market. Eventually, carrier/ligand-mediated targeted cytokines, that deliver cytokines to the target site, should lead to even more effective and less toxic treatments. This strategy has been started in cytokine-based cancer therapy and will become a novel trend in the treatment of many other diseases in the future.

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